UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

MARCONDES SENA FILHO

CORRELAÇÃO DA EXPRESSÃO DOS FATORES DE TRANSCRIÇÃO RUNX1 E ETV5 COM A EXPRESSÃO DAS METALOPROTEINASES 2 E 9 EM LEUCOPLASIAS E CARCINOMAS ESPINOCELULARES BUCAIS

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutor em Estomatopatologia, na Área de Patologia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the dregree of Doctor in Estomatopatologia, in Pathology area.

Orientador: Jacks Jorge Junior

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"O fardo é proporcional às forças, como a recompensa será proporcional à resignação e à coragem."

Allan Kardec

RESUMO

O carcinoma espinocelular (CEC) oral é a neoplasia maligna mais comum da região de cabeça e pescoço. As alterações da mucosa oral que antecedem o desenvolvimento do CEC são de grande interesse neste contexto, no qual a leucoplasia oral é alvo de diversos estudos. Runx1 é um fator de transcrição envolvido em eventos fisiológicos e patológicos da hematopoese, porém suas funções em neoplasias malignas sólidas ainda são pouco conhecidas. Alguns estudos têm sugerido uma possível interação do Runx1 com outros fatores de transcrição, como o ETV5, estimulando a produção de metaloproteinases 2 e 9 em algumas neoplasias solidas, como o carcinoma endometrióide e condrossarcoma. Utilizando imunoistoquímica, imunofluorescência, Western blot e zimografia, o presente estudo avaliou e correlacionou a expressão de Runx1 e ETV5 com a expressão das MMP-2 e MMP-9, integridade da lamina basal e índice de proliferação celular de leucoplasias, carcinomas espinocelulares e mucosas normais orais. Os resultados demostraram que a expressão de Runx1 e ETV5 em leucoplasias apresentando displasia epitelial intensa e CECs orais estão correlacionadas com a alta expressão das MMP-2 e MMP-9, rompimento da lamina basal e alto índice de proliferação celular do parênquima lesional. Diante disso, sugere-se que os fatores de transcrição Runx1 e ETV5 possuem um papel importante do desenvolvimento do carcinoma espinocelular oral.

Palavras-chave: Neoplasia Bucal. Leucoplasia Bucal. Fatores de Transcrição.

ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck region. The mucosal changes that precede the occurrence of OSCC are of great interest in this context, in which the oral leukoplakia (OL) is a subject of several studies. Runx1 is a transcription factor involved in physiological and pathologic events of hematopoiesis, but its functions in solid tumors is still poorly understood. Some studies have suggested a possible interaction of Runx1 with others transcription factors, as ETV5, stimulating the production of matrix metalloproteinases (MMP) 2 and 9 in some solid malignancies, as endometrioid carcinoma and chondrosarcoma. Through immunohistochemistry, immunofluorescence, Western blot and zymography, this study evaluated and correlated the expression of Runx1 and ETV5 with the expression of MMP-2 and MMP-9, epithelial basal membrane integrity and cellular proliferation index in OL, OSCC and normal oral mucosa. The results demonstrated that the expression of Runx1 and ETV5 are correlated with high expression of MMPs 2 and 9, disruption of epithelial basement membrane and high proliferation index in OL with severe epithelial dysplasia and OSCC, which suggest that Runx1 and ETV5 play an important role in the oral squamous cell carcinoma development.

Keywords: Mouth neoplasms. Leukoplakia, oral. Transcription Factors.

Sumário

1 INTRODUÇÃO

Apesar do avanço na prevenção e tratamento, o câncer ocasionou mais de 8 milhões de mortes no mundo em 2013, sendo a segunda colocada dentre as causas de morte dos seres humanos (Fitzmaurice *et al.*, 2015). O CEC oral é uma doença multifatorial e com diversos fatores de risco, como a presença de desordens potencialmente malignas, tabagismo e o etilismo (Hashibe *et al.*, 2009; Scully & Bagan, 2009). Desordens potencialmente malignas (DPMs) são definidas como "um tecido morfologicamente alterado, o qual apresenta maior tendência a desenvolver um carcinoma, quando comparado a um tecido normal". A leucoplasia oral (LO) é a principal representante das DPMs, sendo definida como "placa ou mancha branca que não pode ser caracterizada clinicamente ou patologicamente como qualquer outra enfermidade". Trata-se de um termo clínico usado temporariamente como hipótese diagnóstica, que implica na exclusão de outras lesões de aspecto similar (Gale *et al.*, 2005; Amagasa *et al.*, 2011). A taxa de transformação maligna da LO varia de 0,13% a 34%, sendo a idade, sexo, tipo clínico da lesão e grau de displasia importantes fatores de risco para a malignização (Warnakulasuriya & Ariyawardana, 2015).

A progressão de um carcinoma consiste na multiplicação desenfreada de células tumorais e invasão dos tecidos adjacentes. Para que tal evento ocorra, é necessário que as células cancerosas rompam a lâmina basal que envolve seu tecido de origem, sendo esta uma das primeiras barreiras naturais contra a invasão tumoral (Robinson *et al.*, 2003; de Vicente *et al.*, 2005). Levando-se em conta que a lâmina basal é constituída principalmente por colágeno IV e laminina, estudos têm relacionado à expressão aumentada das metaloproteinases 2 e 9 com o potencial maligno e invasivo de vários tipos de câncer, dentre eles o CEC oral (Bindhu *et al.*, 2006; El Houda Agueznay *et al.*, 2007; Hohberger *et al.*, 2008). As metaloproteinases de matriz (MMPs) são endopeptidases cálcio-dependentes, estruturalmente e funcionalmente semelhantes, responsáveis pela degradação da matriz extracelular, identificadas pela primeira vez em vertebrados em 1962, por Jerome Gross e Charles M. Lapiere (Gross & Lapiere, 1962). Desde então, avanços significativos nas pesquisas demonstraram que a expressão e atividade anormais das MMPs podem estar relacionadas a diversas doenças inflamatórias, malignas e degenerativas (Cawston, 1996; Johnson *et al.*, 1998; Massova *et al.*, 1998).

Translocações cromossômicas estão cada vez mais relacionadas a uma variedade de malignidades do corpo humano. Uma das mais conhecidas e que vem sendo estudada há décadas é a translocação *t*(8;21)(q22;22), frequentemente encontrada no DNA de células leucêmicas de pacientes acometidos pela Leucemia Mieloide Aguda (LMA) do subtipo M2. Desde sua descoberta no inicio dos anos 90, o gene *AML1* teve outros nomes como *PEBPA2B*

e *CBFA2*, porém, em 1999, a Organização Genoma Humano o nomeou formalmente como *fator de transcrição runt-relacionado 1* ("*Runt-related transcription factor 1*"), o *Runx1* (Miyoshi *et al.*, 1991; Speck & Gilliland, 2002). Posteriormente, descobriu-se que tratava-se de uma família de fatores de transcrição com três membros – Runx1, Runx2 e Runx3 – cada um participando de processos fisiológicos e patológicos distintos, por exemplo: o Runx1 está relacionado com a hematopoese e LMA, o Runx2 com a formação óssea e o Runx3 com o sistema imunológico, gastrointestinal e desordens neurais (Chuang *et al.*, 2013).

Há três décadas foi identificado no vírus E26 da eritroblastose aviária um gene que codifica um fator de transcrição, até então nomeado gene "*E26 transformation-specific* (*ETS*)" (Hsu *et al.*, 2004). Atualmente, sabe-se que existem 28 genes *ETS* nos humanos, os quais se dividem em 12 subfamílias, dentre as quais encontra-se o *PEA3*, que codifica os fatores de transcrição ETV1, 4 e 5 (Oh *et al.*, 2012). Alguns destes fatores estão envolvidos no desenvolvimento de metástases e progressão tumoral através da ativação de MMPs e cicloxigenases 2, causando um pior prognóstico em câncer de ovário, colorretal, pulmão e gástrico (Hida *et al.*, 1997; Davidson *et al.*, 2003; Horiuchi *et al.*, 2003; Davidson *et al.*, 2004; Yamamoto *et al.*, 2004; Sloan *et al.*, 2009). Estudos relacionando os *PEA3* em CECs de cabeça e pescoço são escassos, porém foi demostrado que estes genes estão relacionados com a expressão de uma série de MMPs (1, 2, 7, 9, 13 e 14) em CECs esofágicos (Yuen *et al.*, 2011). O ETV5 parece estar relacionado com a produção e ativação da MMP-2 em carcinomas endometriais, conferindo uma maior capacidade de invasão tumoral. Além disso, já foi demonstrado a expressão e a codistribuição das MMPs 2 e 9 com o Runx1 e ETV5 em carcinomas endometrióides e ovarianos, apresentando maior expressão nos frontes de invasão tumoral com degradação da lamina basal (Monge *et al.*, 2007; Furlan *et al.*, 2008; Planaguma *et al.*, 2011).

Supondo-se que a interação entre os fatores de transcrição Runx1 e ETV5 pode estar correlacionada com o aumento da expressão das MMPs 2 e 9 no desenvolvimento e progressão do carcinoma espinocelular oral, o presente estudo avaliou e correlacionou a expressão do Runx1 e ETV5 com a expressão das MMPs 2 e 9, integridade da membrana basal epitelial e índice de proliferação celular em leucoplasias, carcinomas espinocelulares e mucosas normais da cavidade oral.

2 ARTIGO: The transcription factors Runx1 and ETV5 are correlated with high expression of metalloproteinases 2 and 9 in severe oral epithelial dysplasia and oral squamous cell carcinoma

Artigo submetido ao periódico Oncotarget Journal (**ANEXO 2**)

Autoria:

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck region. The mucosal changes that precede the occurrence of OSCC are of great interest in this context, in which the oral leukoplakia (OL) is a subject of several studies. Runx1 is a transcription factor involved in physiological and pathologic events of hematopoiesis, but its functions in solid tumors is still poorly understood. Some studies have suggested a possible interaction of Runx1 with others transcription factors, as ETV5, stimulating the production of matrix metalloproteinases (MMP) 2 and 9 in some solid malignancies, as endometrioid carcinoma and chondrosarcoma. Through immunohistochemistry, immunofluorescence, Western blot and zymography, this study evaluated and correlated the expression of Runx1 and ETV5 with the expression of MMP-2 and MMP-9, epithelial basal membrane integrity and cellular proliferation index in OL, OSCC and normal oral mucosa. The results demonstrated that the expression of Runx1 and ETV5 are correlated with high expression of MMPs 2 and 9, disruption of epithelial basement membrane and high proliferation index in OL with severe epithelial dysplasia and OSCC, which suggest that Runx1 and ETV5 play an important role in the oral squamous cell carcinoma development.

Keywords: Oral Carcinoma, Oral Leukoplakia, Potentially Malignant Disorders, AML1, ETS

INTRODUCTION

Despite substantial progress in prevention and treatment, cancer is the second leading cause of death, causing over 8 million deaths worldwide in 2013. Oral squamous cell carcinoma (OSCC) is the most prevalent malignancy of the head and neck [1, 2]. The transition from normal mucosa to invasive carcinoma is complex and involves a multistep and multifactorial etiology, in which mucosal changes that precede the occurrence of OSCC are of great interest [3, 4]. The presence of potentially malignant disorders (PMD) is one of most important risk factors for the development of OSCC. Tobacco smoking and alcohol consumption have a synergistic effect and are the main etiological factors of PMD and OSCC [5, 6]. PMD comprise lesions and conditions that present morphological alterations with increased potential for malignant transformation, in which oral leukoplakia (OL) is the main lesion. Oral leukoplakia (OL) is "a predominantly white patch or plaque that cannot be characterized clinically or pathologically as any other disorder". Clinically, there are two types of leukoplakia: homogeneous and non-homogeneous. The histopathological diagnosis of leukoplakia is graded according with the presence and degree of epithelial dysplasia: Hyperkeratosis and Acantosis, Mild Epithelial Dysplasia, Moderate Epithelial Dysplasia, Severe Epithelial Dysplasia and Carcinoma in situ [7]. Despite variability of study results, the malignant transformation rate of OL varies from 0.13 to 34%, in which age, gender, clinical type of the lesion and grade of dysplasia seem to be important risk factors [8]. Several proteic, genetic and molecular studies have tried to understand the development process of PMDs, aiming to find a predictive biomarker for its malignization.

Runt-related transcription factor (Runx) protein is a DNA-binding subunit of heterodimeric transcription factor CBF (Core Binding Factor) [9]. Runx proteins have a DNA binding domain in their N-terminal portion, which consists of 128-amino-acid domain [10]. In mammals, there are three Runx proteins: Runx1, Runx2, and Runx3. The first (Runx1) is associated with multiple hematopoietic lineages; Runx2 is implicated in cartilage and bone development and the latter (Runx3) is associated with immunity and inflammation [11, 12]. Runx1 is an hematopoietic stem cell factor expressed in all hematopoietic cell lineages with the exception of mature erythroid cells, playing a critical role for hematopoietic development [13]. Recently, Runx1 was associated with some epithelial neoplasias, such as breast cancer [14, 15], colon and rectal cancers [16], prostate cancer [17, 18] and endometrial and ovarian cancers [19, 20]. ETV5 (ETS Transcript Variant 5, also called ERM for ETS-related molecule) is a transcription factor, member of the subfamily PEA3 [21]. It is involved in reproduction and fertility, kidney and epithelium development [21], esophagic squamous cell carcinoma [22], chondrosarcoma [23] and endometrial cancer [24]. A possible interaction

between Runx1 and ETV5 with intra and extra-cellular events, culminating in neoplastic progression and invasion in skin squamous cell carcinoma and endometrial carcinomas has been suggested. Furthermore, it was suggested that Runx1 and ETV5 stimulate the production of metalloproteinases 2 and 9 in solid malignancies [25].

Matrix metalloproteinases (MMP) are calcium-dependent endopeptidases responsible for physiological and pathologic degradation of the extracellular matrix (ECM). The constitutive expression levels of MMPs are usually low, being altered only in some physiological circumstances when the ECM needs remodeling, as in embryogenesis and bone remodeling. Since their discovery, studies have shown that the abnormal expression and activity of MMPs may be related to some inflammatory, malignant and degenerative diseases [26-28]. MMPs have been extensively studied in a range of malignancies including lung, gastric, breast, ovarian and oral carcinomas. MMPs are produced by surrounding and malignant cells, contributing to proliferation, invasion and metastasis. The basal membrane (BM) that surrounds the original tissue is one of the first barriers that neoplastic cells have to overcome in this process [29-33]. The BM is mostly composed by type IV collagen and laminin, the main types of collagen degraded by MMP-2 and -9 [34-36]. Therefore, several studies have suggested that these MMPs are highly expressed and active in PMDs and OSCC, culminating in poor prognosis [29, 37, 38].

Until now, no studies have characterized and correlated the expression of Runx1 and ETV5 in OL and OSCC. It is suspected that the interaction between Runx1, ETV5, MMP-2 and MMP-9 may be related to malignant transformation of OL and progression of OSCC. Therefore, this study evaluated and correlated the expression of transcription factors Runx1 and ETV5 with the expression of metalloproteinases 2 and 9 in OL, OSCC and normal oral mucosa. Furthermore, the results obtained were correlated with the epithelial BM integrity and cellular proliferation index.

RESULTS

Clinicopathological characteristics

Fresh samples from incisional biopsies performed in lesions with OSCC and/or OL clinical suspicion were collected from 56 patients (31 males and 25 females). The clinico-pathological characteristics of the lesions are summarized in **Table 1**. As control, samples of normal oral mucosa from 16 patients (9 males and 7 females) were collected. These patients not reported smoking and/or alcohol consumption habits and had non-cancer related lesions. The mean age of control group was 56.7 years (range 34 – 88 years) and samples were collected from the buccal mucosa (7), alveolar rebord (5) and tongue (4).

Clinico-pathological characteristics	
Gender, n (%)	
Male	31 (55.4)
Female	25 (44.6)
Age (years), mean (range)	63.2 (33–89)
Smoking, n (%)	
Non-smoker	18 (32)
Current smoker	28 (50)
Ex-smoker	10 (18)
Alcohol Consumption, n (%)	
Non-consumers	37 (66)
Current consumer	12 (21)
Ex-drinker	7 (13)
Lesion Location, n (%)	
Tongue	19 (34)
Alveolar Rebord	13 (23)
Soft palate	8 (14)
Floor of mouth	7 (12.5)
Buccal mucosa	6 (11)
Gingiva	2 (3.5)
Hard palate	1 (2)
Clinical Diagnosis, n (%)	
Homogeneous OL	13 (23)
Non-homogeneous OL	25 (45)
OSCC	18 (32)
Microscopic Diagnosis, n (%)	
HA	12 (21.5)
MMD	17 (30)
SD	12 (21.5)
SCC	15 (27)

Table 1 – Clinicopathological characteristics of the patients included in present study. Legends: Oral Leukoplakia (**OL**), Oral Squamous Cell Carcinoma (**OSCC**), Hyperkeratosis and Acanthosis (**HA**), Mild/Moderate Epithelial Dysplasia (**MMD**), Severe Epithelial Dysplasia (**SD**), Mild/Moderately Differentiated Squamous Cell Carcinoma (**SCC**).

The transcription factors Runx1 and ETV5 are expressed in oral severe epithelial dysplasia and oral squamous cell carcinoma

The expression levels of Runx1 and ETV5 were measured by Western blot (**Figure 1A**). A weak expression of both factors in was observed in Control, HA and MMD samples. SD and SCC samples presented increased expression of Runx1 and ETV5 ($p<0.001$ and $p=0.001$, respectively, **Figure 1B**). Additionally, there was a strong and positive monotonic correlation between Runx1 and ETV5 expression levels ($r_s=0.721$, $n=72$, $p<0.0001$). The samples from ex-drinkers patients presented a significantly increased expression of Runx1 and ETV5 ($p=0.01$ and $p=0.005$, respectively). However, samples from current alcohol and tobacco-consuming patients presented significantly increased expression of Runx1 only ($p=0.017$).

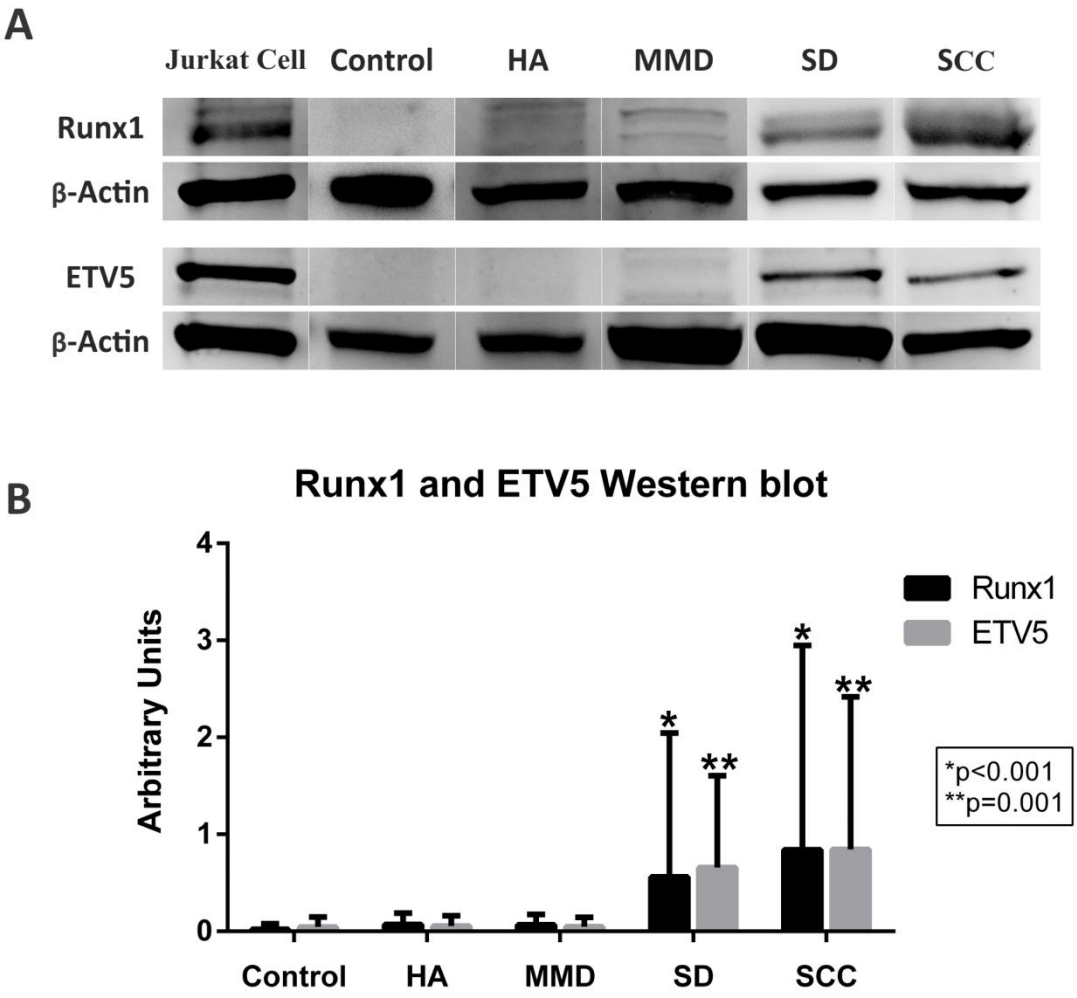

Figure 1 - Runx1 and ETV5 Western blot. (**A**) Representative Western blot for Runx1 (~55 kDa) and ETV5 (~58 kDa) for each microscopic diagnosis with Jurkat Cell Lysate as positive control. All samples are with 30µg of protein extractions. (**B**) Western blot analysis showed weakly expression of Runx1 and ETV5 in Control, HA and MMD, but was significantly increased in SD and SCC samples. Data are represented as mean with Standard Deviation.

In immunostaining assays, the expression of both factors was mainly localized in the parenchymal cells, but a weak and sparse immunoreactivity in the stromal compartment with inflammatory infiltration was observed. Furthermore, variable immunoreactivity in glandular and vascular cells was observed. Runx1 was expressed in the parenchyma of HA, MMD, SD and SCC, while ETV5 was expressed only in SD and SCC. No evident immunoreactivity in the parenchyma of control samples for both factors was observed (**Figure 2A and B**). Therefore, the expression of Runx1 in control samples and ETV5 in control, HA and MMD samples detected in the Western blot was considered as a product of an inflamed stroma and glandular and vascular structures. Runx1 was weakly expressed in some basal epithelial cells in HA and diffusely expressed in 2/3 of epithelium in some MMD samples (**Figure 2C and** E). A strong expression of Runx1 and ETV5 was observed in SD (**Figure 2G and H**) and SCC samples (**Figure 3**). It was observed that the expression of both factors did not occur in the whole of SCC parenchyma, but mostly in superficial tumoral islands (**Figure 3A and B**). At the cellular level, Runx1 was found diffusely in the epithelial cytoplasm and weakly in the nucleus, while ETV5 was expressed weakly in the cytoplasm and strongly in the nucleus (**Figure 3C and D**). Both factors presented a granular staining pattern (**Figure 3E and F**).

Samples that showed some level of transcript factor expression in Western blot and positive immunoreactivity in the lesional parenchyma were considered positive for Runx1 or ETV5. The results are summarized in **Figure 4A**. The co-expression of Runx1 and ETV5 was evaluated by double immunofluorescence. It was observed that 58.3% of SD and 53.3% of SCC co-expressed Runx1 and ETV5 (**Figure 4B**). The immunofluorescence assay demonstrated more evidently the granular staining pattern in the cytoplasm and nucleus of Runx1 and ETV5. The co-localization was observed in cytoplasm and more prominent in nucleus of parenchymal epithelial cells (**Figure 5 and 6**).

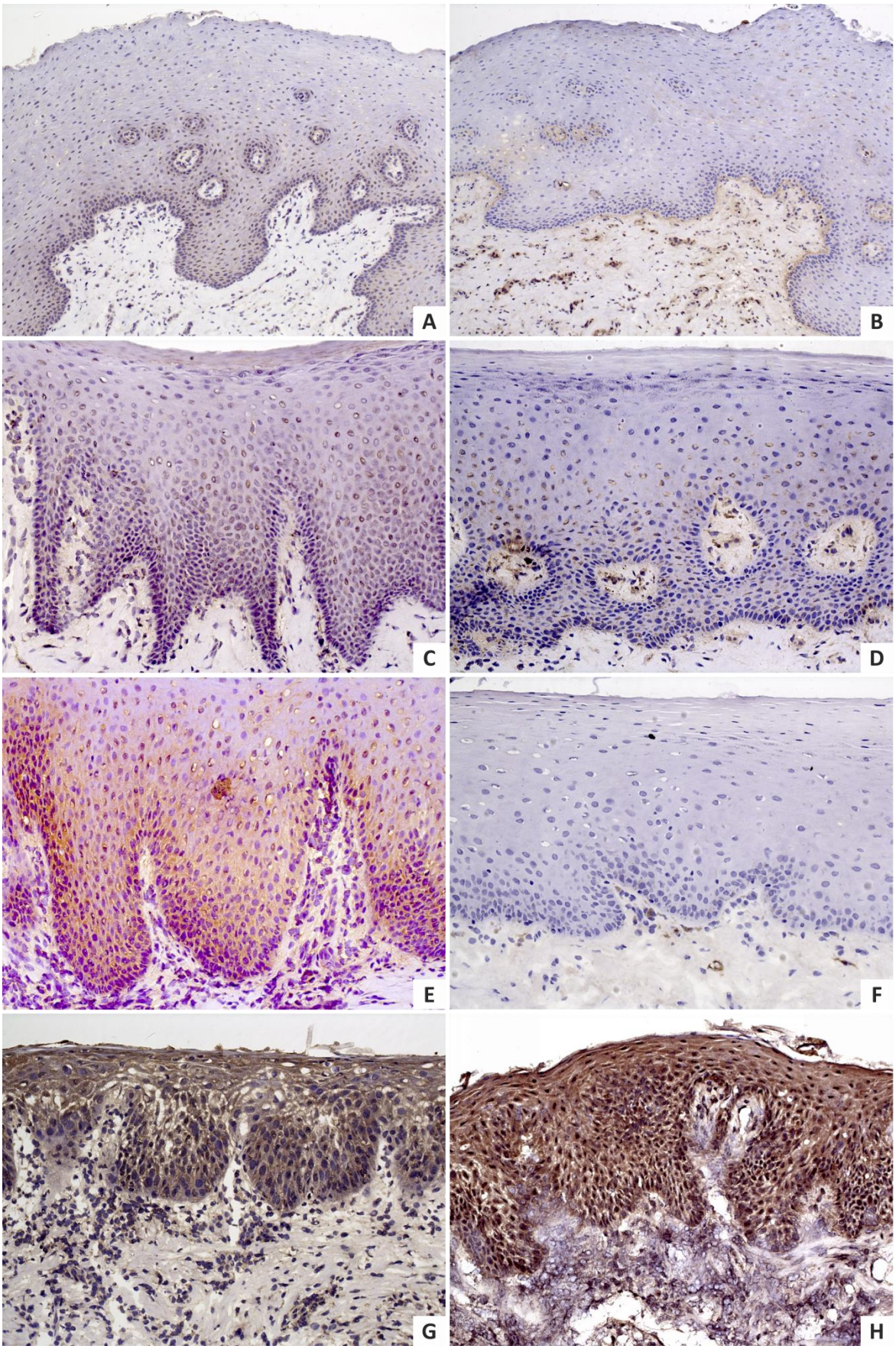

Figure 2 - Runx1 and ETV5 immunohistochemistry in OL samples. Immunoreactivity in epithelium of control samples for Runx1 (**A, 100x**) and ETV5 (**B, 100x**) were not observed. Runx1 was weakly expressed in some basal cells of HA (**C, 200x**) and in 2/3 of epithelium of some MMD samples (**E, 200x**). ETV5 was not expressed in HA (**D, 200x**) and MMD (**F, 200x**). Strong cytoplasmic expression of Runx1 and strong nuclear expression of ETV5 were observed in some cases of SD (**G** and **H**, respectively, **200x**).

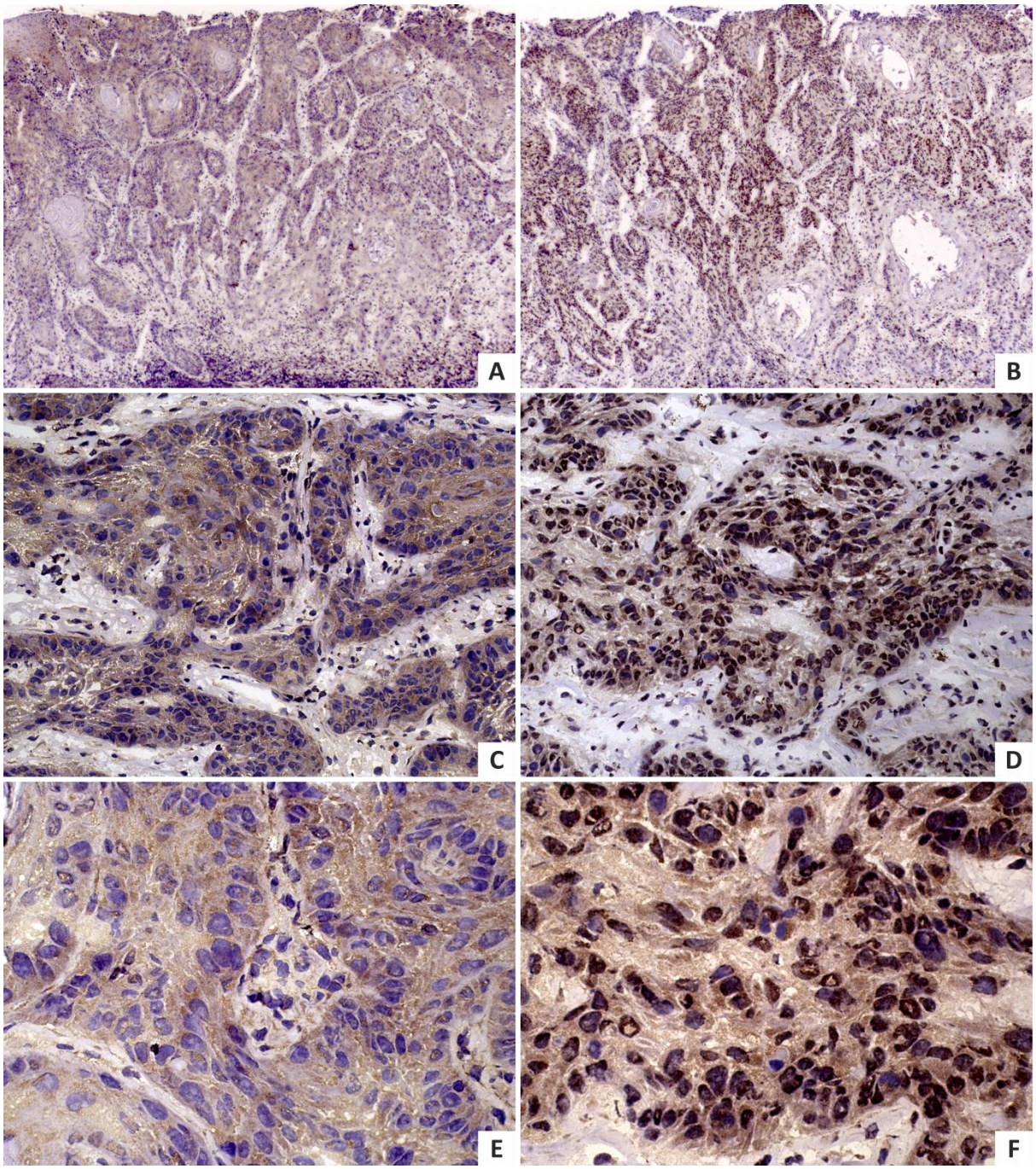

Figure 3 - Runx1 and ETV5 immunohistochemistry in SCC sample. The expression of both transcription factors did not occur in the whole parenchyma, but mostly in superficial tumoral islands (**A** and **B**, respectively**, 50x**). At the cellular level, Runx1 was found diffusely in epithelial cytoplasm and weakly in the nucleus, while ETV5 was weakly expressed in the cytoplasm and strongly expressed in the nucleus (**C** and **D**, respectively, **200x**). Both markers presented a dot-like and granular staining pattern (**E** and **F**, **400x**).

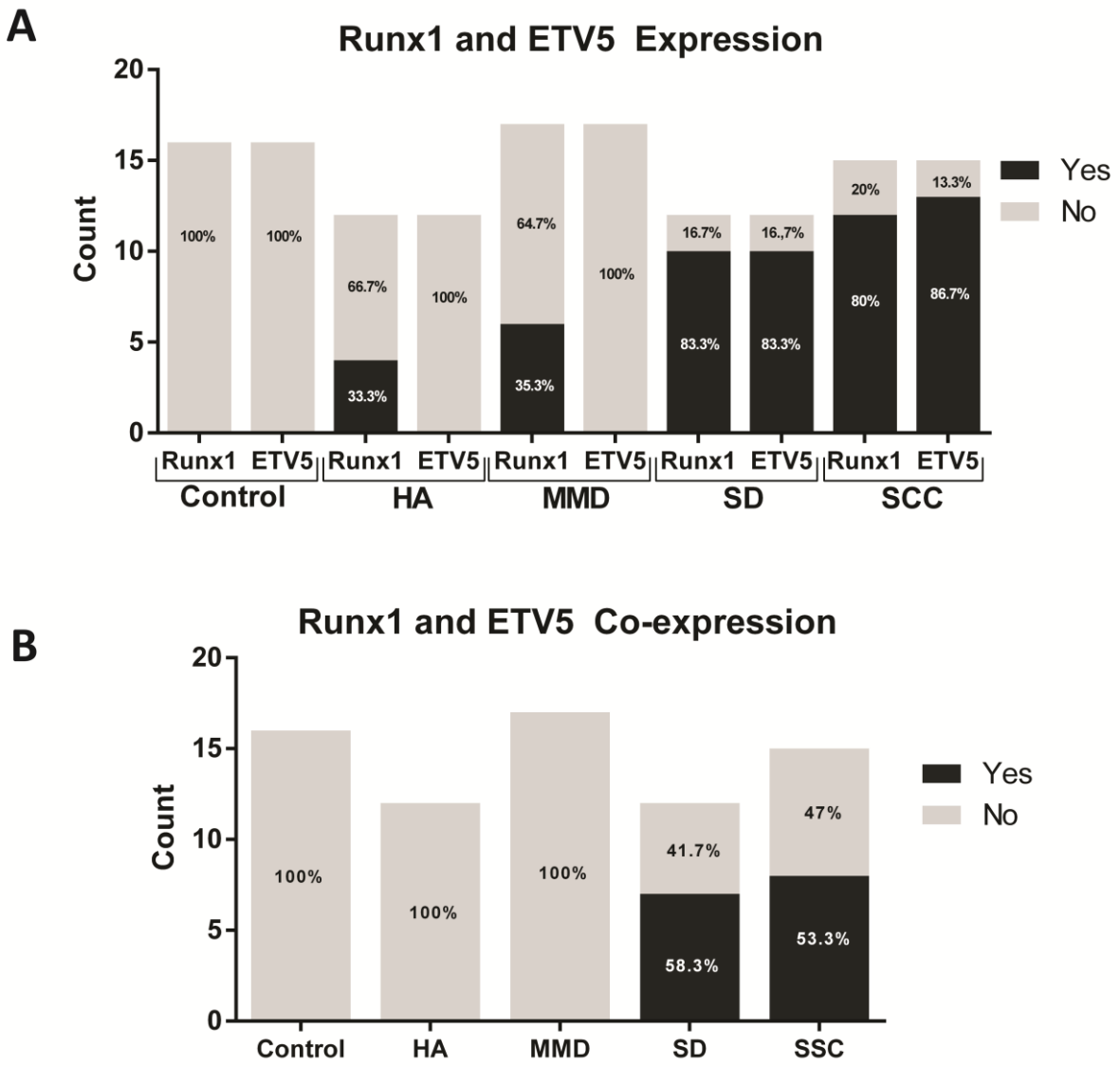

Figure 4 - Expression and Co-expression of transcriptions factors Runx1 and ETV5. Number and percentage of samples that expressed Runx1 and ETV5 individually (**A**) and co-expressed in the parenchyma (**B**).

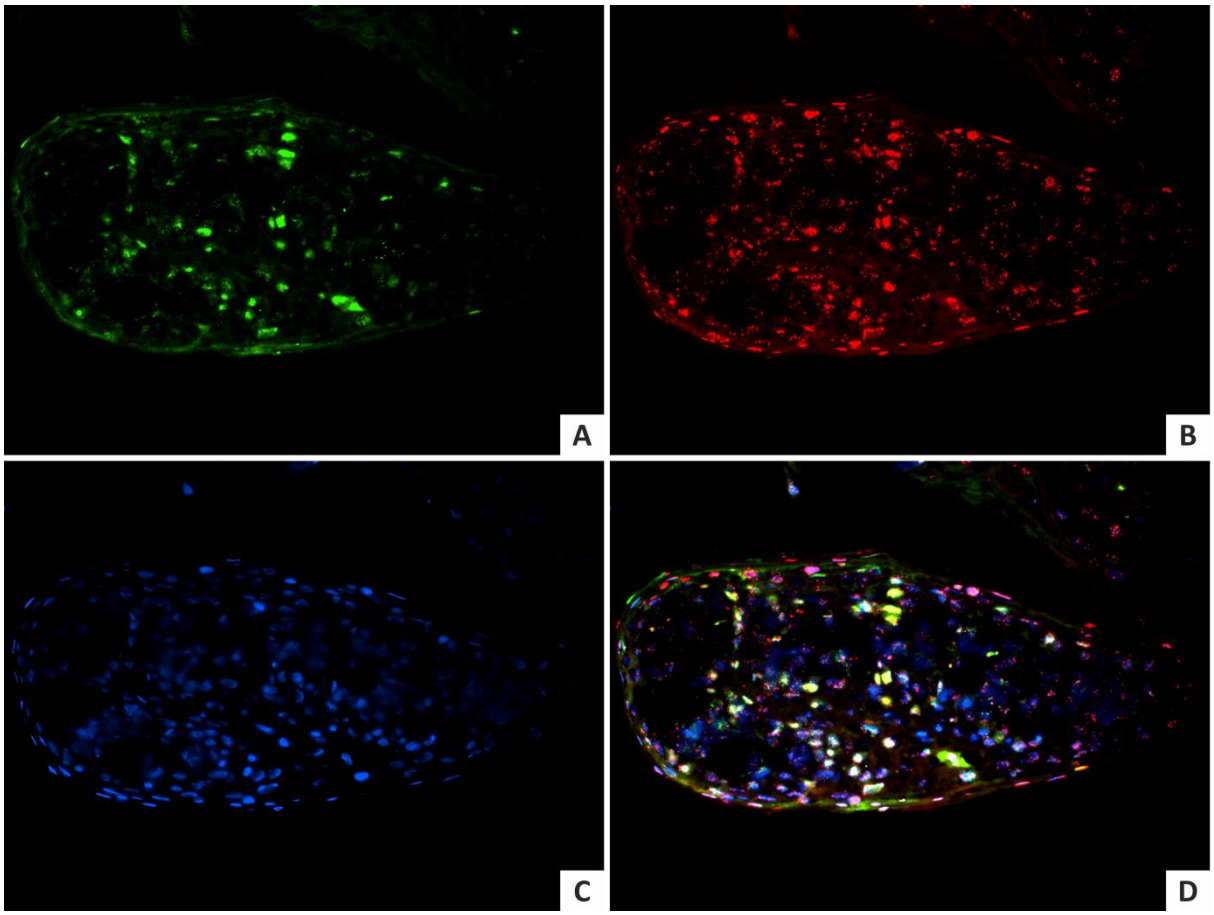

Figure 5 - Runx1 and ETV5 double immunofluorescence in SCC sample (200x). The immunofluorescence detection of Runx1 (**A**, **Green**) and ETV5 (**B**, **Red**) confirmed the results of Western blot and immunostainings. The epithelial nuclei were counterstained with fluorescent stain DAPI (**C**, **Blue**). The merged images show the co-expression of Runx1 and ETV5 (**D**). The co-localization occurred mainly in the cell nucleus, where it is shown as white color points.

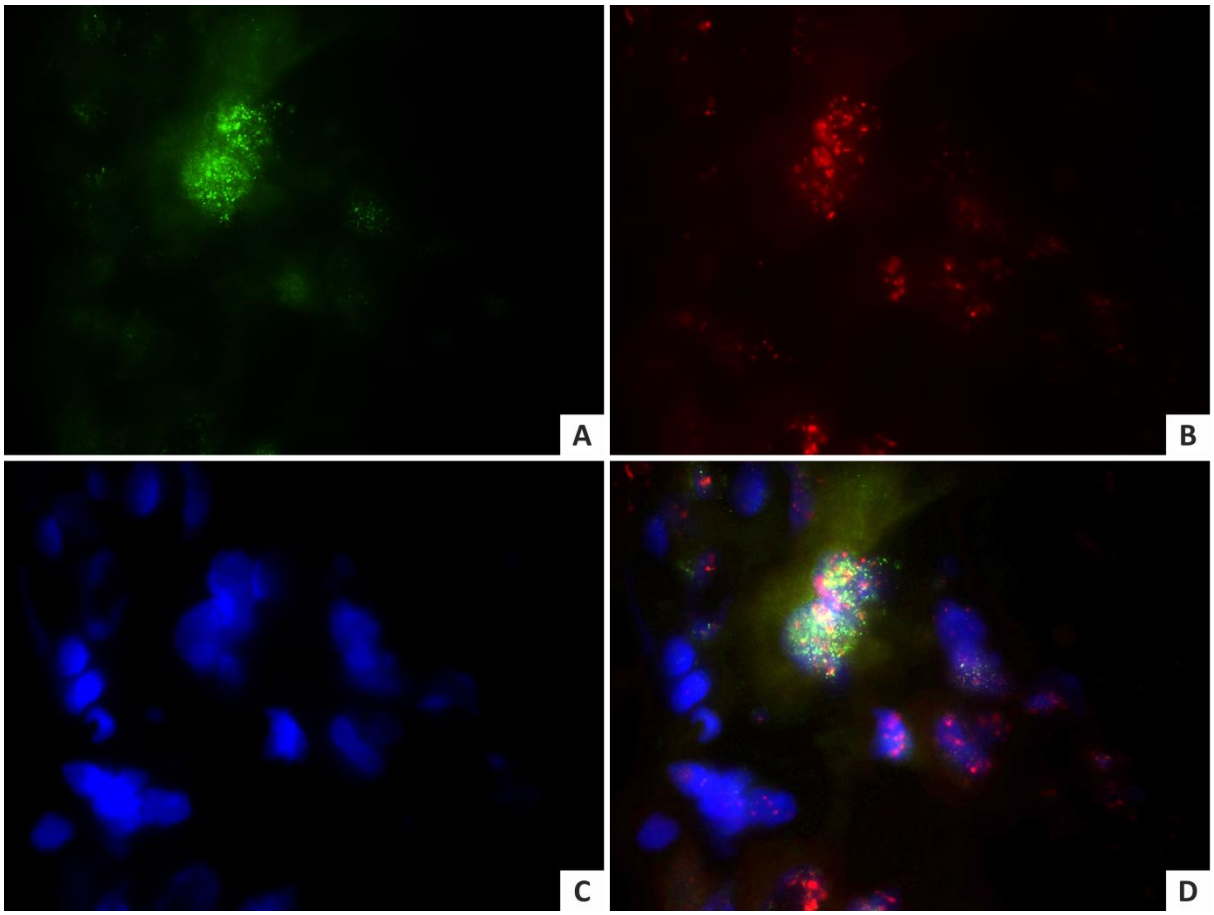

Figure 6 - Runx1 and ETV5 double immunofluorescence in SCC sample (1000x). The immunofluorescences demonstrated the evident granular and dot-like staining pattern of Runx1 (**A**, **Green**) and ETV5 (**B**, **Red**) in the cytoplasm and mainly in nucleus (**C**, **Blue**) of parenchymal cells. The merged images show the co-expression of Runx1 and ETV5 (**D**). The co-localization occurred mainly in the cell nucleus, where it is shown as white color points.

The samples that co-expressed Runx1 and ETV5 presented increased expression of MMP-2 and MMP-9

The expression of MMPs 2 and 9 was measured by their gelatinolytic activity in zymography. All samples expressed pro and activated forms of MMP-2, but only the activated form of MMP-9 (**Figure 7A**). Therefore, the expression of MMP-2 was determined by sum of densitometry values obtained from its pro and activated form bands. MMP-9 expression was positively correlated with the progression of epithelial dysplasia ($r_s=0.564$, $n=72$, $p<0.0001$), being highly expressed in SCC ($p<0.0001$). MMP-2 was constantly expressed in all samples (**Figure 7B**). The samples from smokers expressed significantly more MMP-9 than non-smokers ($p=0.002$). There was a weak positive monotonic correlation between Runx1 and MMP-9 expression levels ($r_s=0.391$, $n=72$, $p=0.001$) and a moderate positive monotonic correlation between ETV5 and MMP-9 ($r_s=0.465$, $n=72$, $p<0.0001$). Furthermore, considering only the presence of Runx1 and ETV5, but not the expression levels, HA and SCC samples expressed more MMP-2 ($p=0.012$) and MMP-9 ($p<0.0001$), respectively, when Runx1 was expressed (**Figure 8A**). When ETV5 was expressed, SCC samples expressed significantly more MMP-9 ($p<0.0001$, **Figure 8B**). Furthermore, when the samples showed co-expression of Runx1 and ETV5, SCC samples expressed significantly more MMP-2 and MMP-9 ($p=0.039$ and $p<0.0001$, respectively, **Figure 8C**).

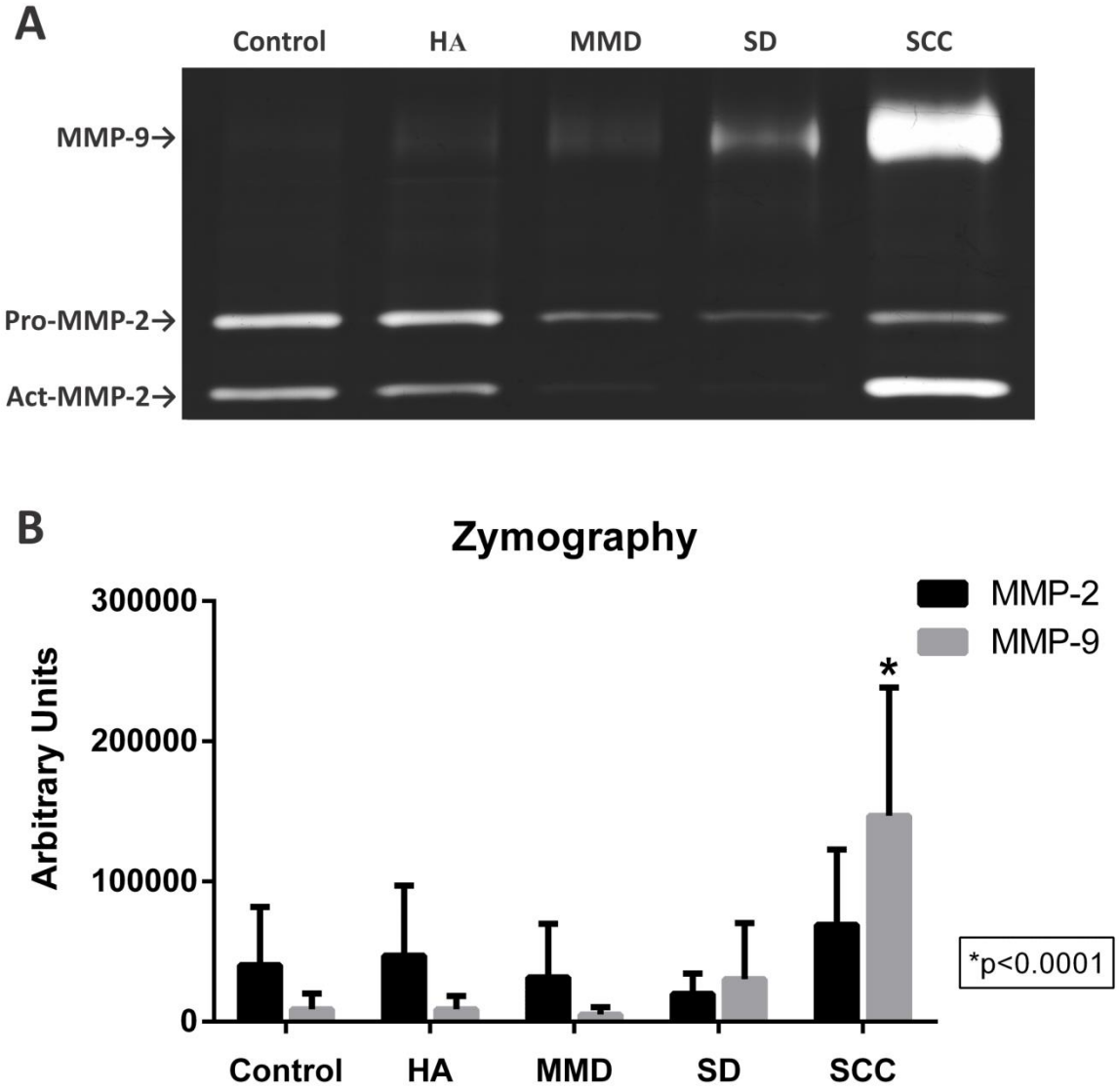

Figure 7 - Zymography. Representative zymography for each sample group, where MMP-2 was found in pro and activated (Act) forms, but MMP-9 was found in activated form only (**A**). The expression of MMP-2 was determined by the sum of densitometry values obtained from its pro and activated form bands. MMP-9 expression was increased according with the progression of epithelial dysplasia, being highly expressed in SCC ($p<0.0001$). The MMP-2 was constantly expressed in all samples (**B**). Data are represented as mean with Standard Deviation.

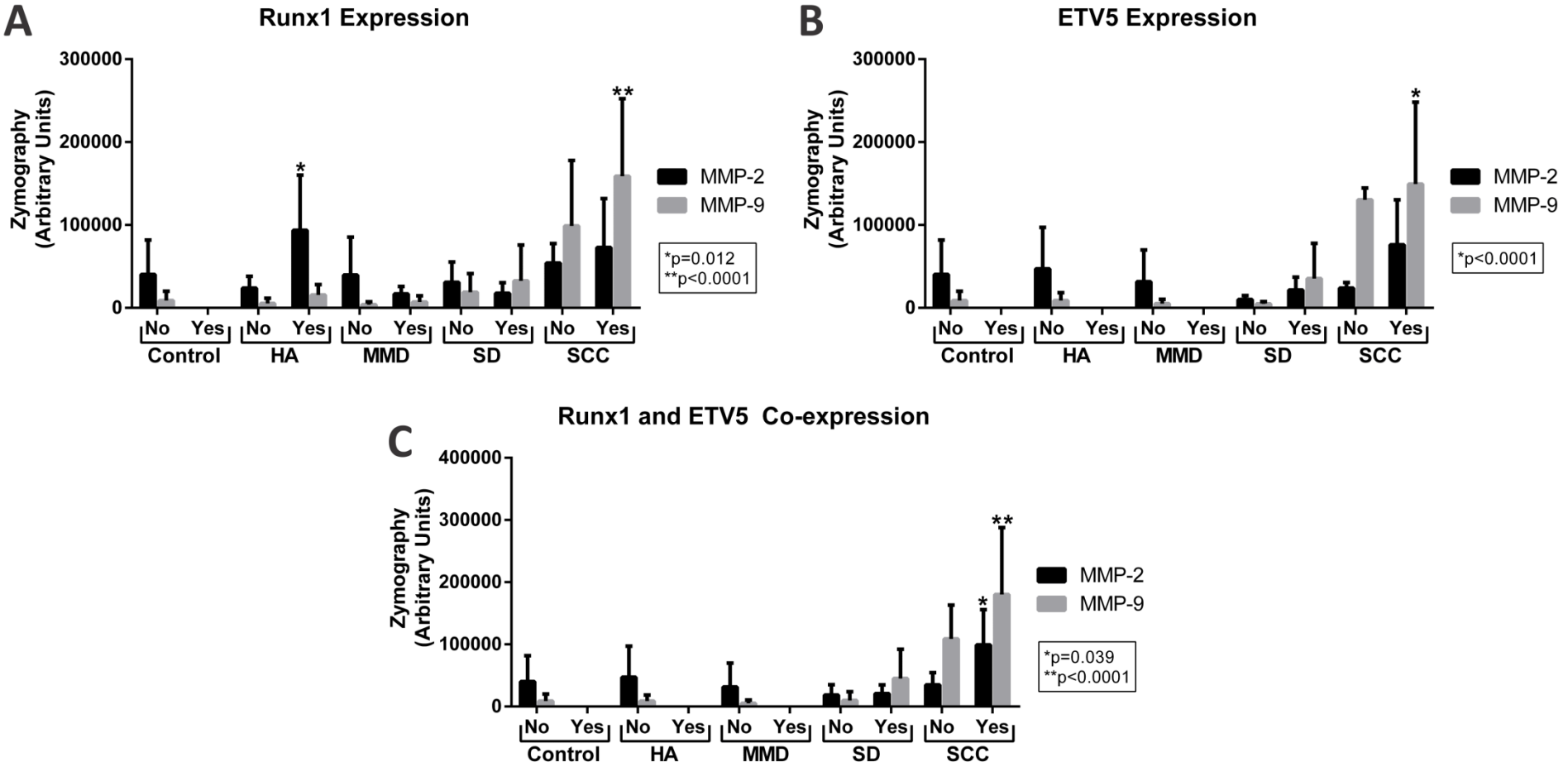

Figure 8 - Correlation between the presence of transcription factors and MMP levels in the samples (data are represented as mean with Standard Deviation). Considering only the presence of Runx1 and ETV5, but not the expression levels, HA and SCC samples expressed more MMP-2 ($p=0.012$) and MMP-9 ($p<0.0001$), respectively, when Runx1 was expressed (**A**). When ETV5 was expressed, SCC samples expressed significantly more MMP-9 ($p<0.0001$, **B**). Furthermore, when the samples showed co-expression of Runx1 and ETV5, the SCC samples expressed significantly more MMP-2 and MMP-9 ($p=0.039$ and $p<0.0001$, respectively, **C**)

In immunostaining assay, MMP-2 and MMP-9 expression was mainly cytoplasmic and diffuse. MMP-2 was strongly expressed in the epithelium of control and HA samples (**Figure 9A and C**), while MMD and SD samples expressed MMP-2 and MMP-9 moderately (**Figure 9E and G**). MMP-9 was weakly expressed in some points of basal layer cells of control and OL samples (**Figure 9B, D, F and H**). SCC samples expressed both MMPs. Whereas MMP-2 was found in some tumoral nests (Figure 10A and B), MMP-9 showed a diffuse and strong expression in some points of parenchyma and around of the tumoral nests with prominent inflammatory infiltrate (Figure 10C and D).

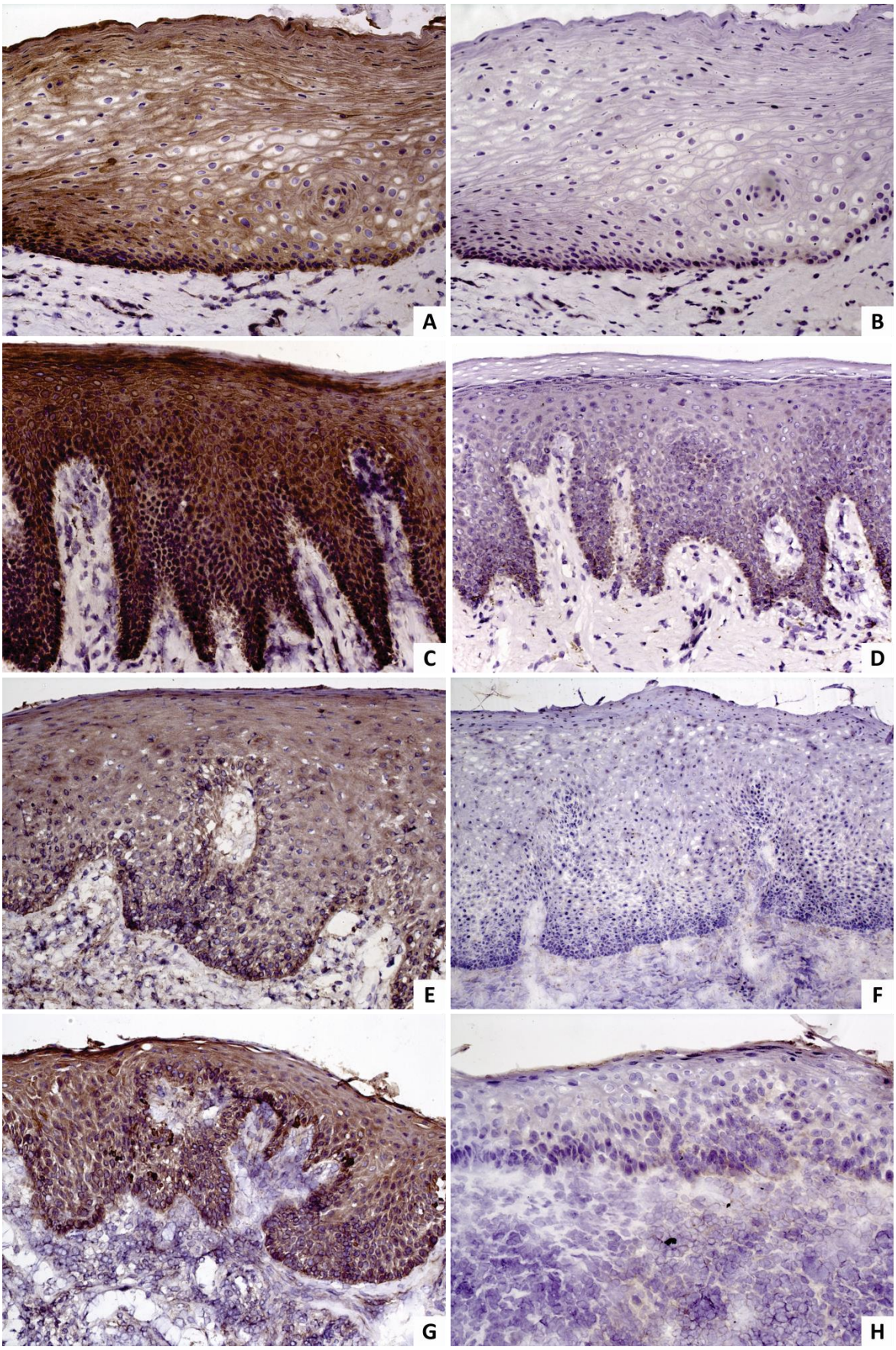

Figure 9 - MMP-2 and -9 immunohistochemistry in OL samples (200x). MMP-2 and MMP-9 expression was mainly cytoplasmic and diffuse. MMP-2 was strongly expressed in the epithelium of control and HA samples (**A** and **C**, respectively), while MMD and SD samples were moderately expressed (**E** and **G**, respectively). MMP-9 was weakly expressed in some points of basal layer cells of control, HA, MMD and SD samples (**B**, **D**, **F** and **H**, respectively).

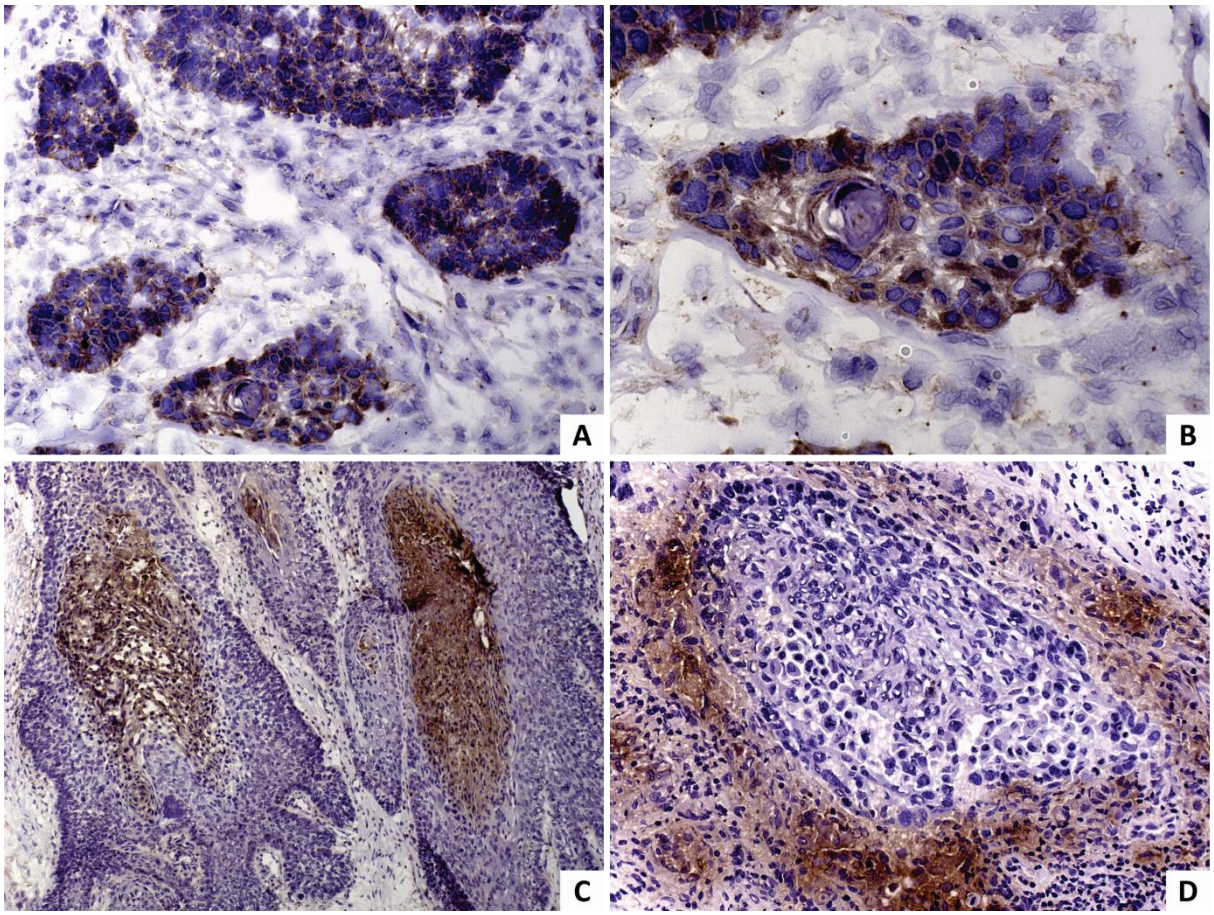

Figure 10 - MMP-2 and -9 immunohistochemistry in SCC sample. SCC samples expressed both MMPs, with MMP-2 found in some tumoral nests (**A**, 200x and **B**, 400x). MMP-9 showed a diffuse and strong expression in some points of parenchyma and around of the tumoral nests with prominent inflammatory infiltrate (**C**, 100x and **D**, 200x).

The Runx1 and ETV5 expression is correlated with discontinuous epithelial basal membrane and increased cellular proliferation index

The integrity of epithelial BM expression was evaluated trough type IV collagen immunofluorescence and the cellular proliferation index was determined by Ki-67 quantification. Half of SD and all SCC samples presented points of discontinuous BM (**Figure 11 and 12A**). These samples expressed significantly more Runx1 and ETV5 ($p<0.0001$, Figure 12B); however, only SCC presented a significantly increased expression of MMP-2 and MMP-9 ($p=0.039$ and $p<0.0001$, respectively, Figure 12C). A progressive increase of cellular proliferation index was observed, according with microscopic diagnosis of the samples ($rs=0.574$, $n=72$, $p<0.0001$), with SCC being significantly higher ($p<0.001$, **Figure 13 and 14**). There was a weak positive monotonic correlation between Runx1 and ETV5 expression levels and cellular proliferation index ($rs=0.306$ and $rs=0.306$, $p=0.009$ and $p=0.002$, respectively, $n=72$), while there was a moderate and positive monotonic correlation between MMP-9 expression levels and cellular proliferation index ($rs=0.426$, $n=72$,

p<0.0001). Additionally, there was a moderate and positive monotonic correlation between the samples that co-expressed Runx1 and ETV5 and cellular proliferation index (rs=0.416, n=72, p<0.0001).

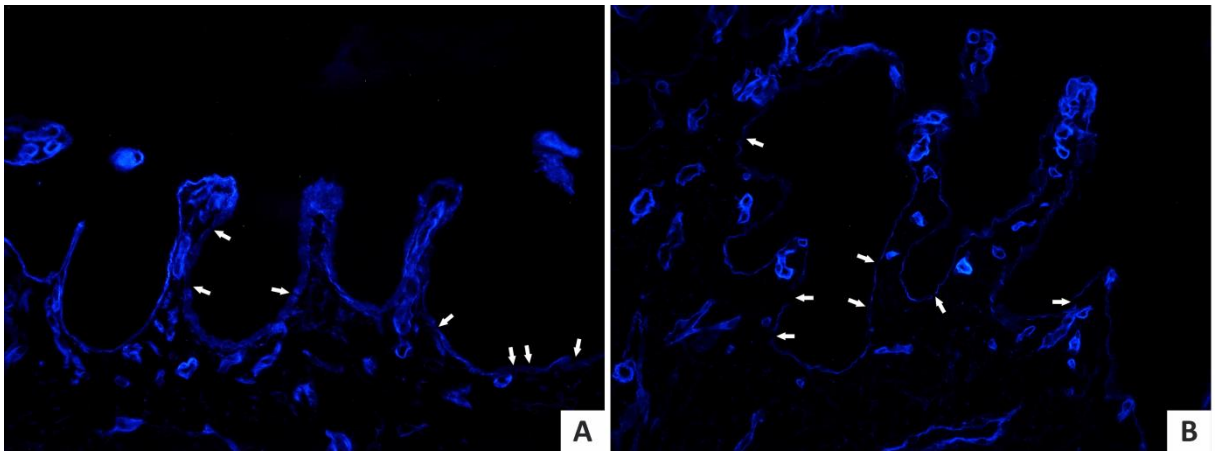

Figure 11 - Type IV collagen immunofluorescence (200x). The integrity of epithelial BM expression was evaluated trough type IV collagen immunofluorescence, and only SD and SCC samples presented points of discontinuous BM (**A** and **B**, respectively) pointed by **white arrows**.

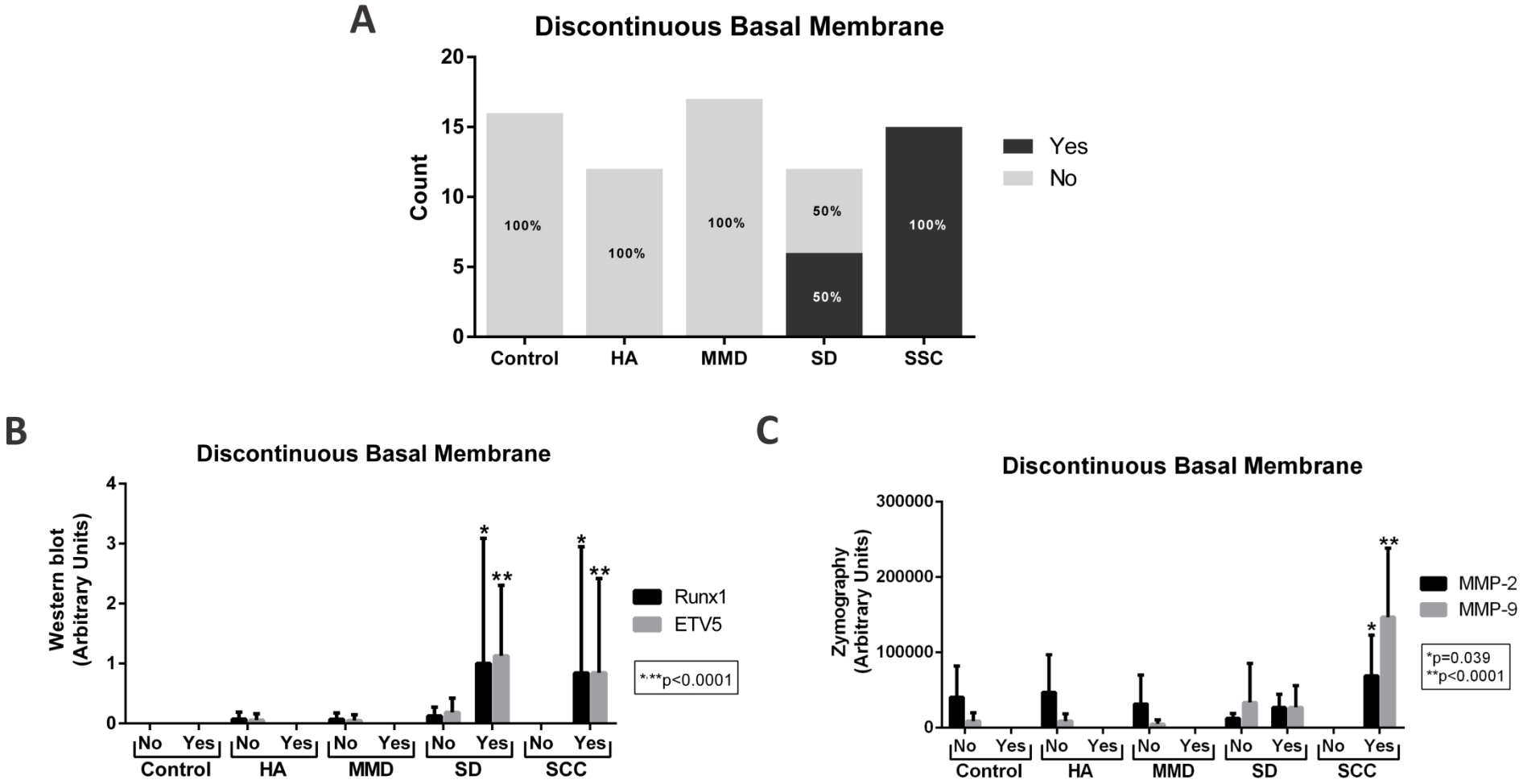

Figure 12 - Integrity of discontinuous basal membrane evaluation and correlation with Runx1, ETV5, MMP-2 and MMP-9 expression levels (data are represented as mean with Standard Deviation). Half of SD and all SCC samples presented points of discontinuous BM (**A**). These samples expressed significantly more Runx1 and ETV5 ($p<0.0001$, **B**) than others, however only SCC presented significantly higher expression of MMP-2 and -9 ($p=0.039$ and $p<0.0001$, respectively, **C**).

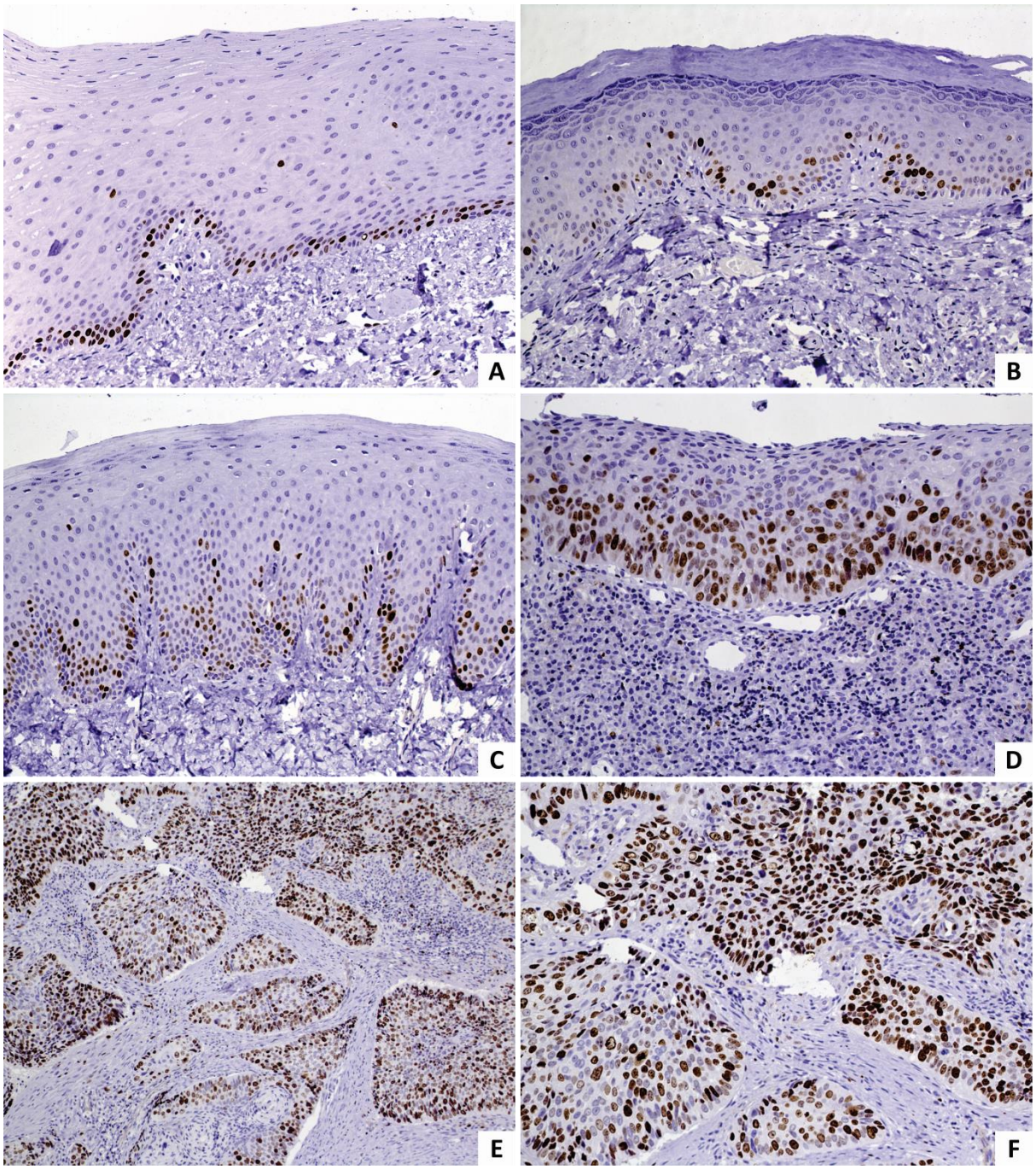

Figure 13 - Ki-67 immunohistochemistry. The cellular proliferation index was determined by Ki-67 quantification. Control and HA samples expressed Ki-67 in some cells of epithelial basal layer (**A** and **B**, respectively, **200x**). An increased expression in the epithelial basal layer of MMD samples was observed (**C**, **200x**). SD samples showed expression of Ki-67 in at least 2/3 of the dysplastic epithelium (**D**, **200x**). High expression of Ki-67 in parenchymal SCC samples was observed (**E**, 100x and **F**, **200x**).

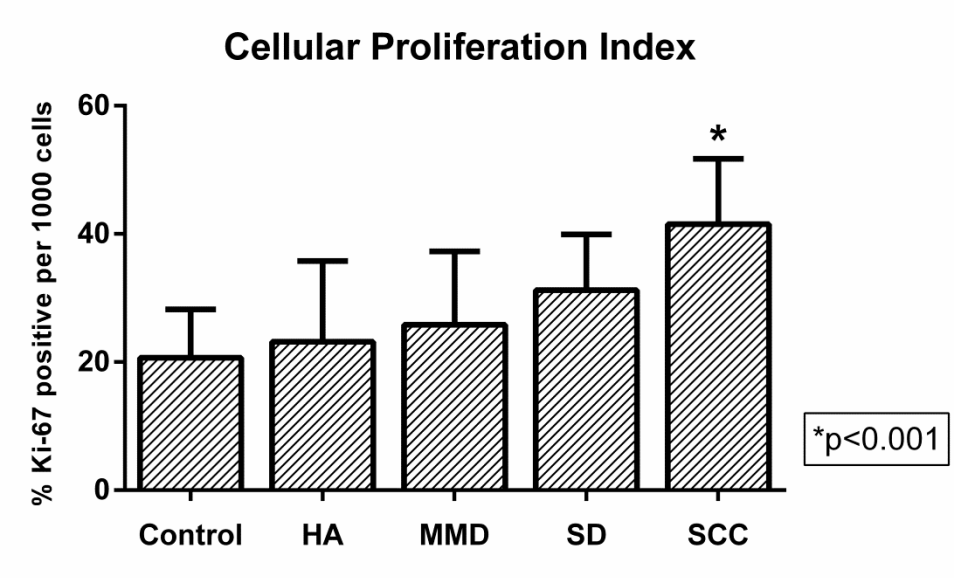

Figure 14 - Cellular proliferation index (data are represented as mean with Standard Deviation). Progressive increase of cellular proliferation index according with microscopic diagnosis of the samples was observed, where SCC was significantly increased ($p<0.001$).

DISCUSSION

Since the discovery of the *Runx1* gene at *t*(8;21)(q22;q22) chromosomal translocation in acute myeloid leukemia (AML) over 40 years, Runx1 is recognized as a human leukaemia and haematopoietic stem cell factor [39]. It was observed several functions of Runx1 in hematopoietic malignancies through interaction with other transcription factors, acting as an oncogene or as a transcriptional suppressor in determined situations [40]. ETV5 is a transcription factor, member of the PEA3 subfamily, one of the 12 subfamilies of ETS transcription factors. PEA3 expression was detected in human solid tumours for the first time by Trimble et al. (1993) in breast cancer, whose results were reinforced by Shepherd et al. (2001) [41, 42]. Besides ETV5, the PEA3 subfamily is composed by ETV1 and ETV4, which are involved in chromosomal translocations in some cases of Ewing sarcoma [43].

Recently, some studies have demonstrated the expression and functions of Runx1 in normal and neoplastic epithelial tissues. Dr. Tumbar and her research group have studied skin stem cells and hair follicle development, performing many discoveries about Runx1 in epithelial squamous cells [25, 44-51]. From this research group, Scheitz et al. published in 2012 a study that characterized the expression and some functions of Runx1 in epithelial squamous cells, including the mucosa of oral cavity. They demonstrated that Runx1 is one of the 10% highly expressed genes in 1/3 of all 138 applicable microarrays studies of human cancers of Oncomine database. Runx1 was found to be overexpressed in blood, brain, breast, lung, pancreas, renal, esophageal and oral malignancies. This group was the first – and only until now – to confirm the high expression of Runx1 by Western blot and immunofluorescence in OSCC cell lines (SCC66, SCC74 and SCC125) in primary OSCC tumours by immunofluorescence, as well as to demonstrate the possible functions of Runx1 [10].

Approximately 80% of SD and SCC samples showed high expression of Runx1 and ETV5, achieving a statistically significant difference versus the others samples in Western blot analysis. Moreover, immunohistochemical expression did not occur in the entire parenchyma, being more common in severe epithelial dysplasias and/or superficial OSCC regions. Scheitz et al. (2012) showed that primary OSCC expressed Runx1 mainly in the proliferative edge cells, which was frequently concomitant with areas Ki-67 expression. Furthermore, they demonstrated that OSCC depends upon Runx1 for normal initiation and growth [10]. In the present study, a significant positive correlation between expression levels of Runx1 and ETV5 and Cellular Proliferation index was observed, suggesting that lesions with greater proliferative potential tend to express more Runx1 and ETV5. Therefore, it is suggested that Runx1 and ETV5 are correlated with the initiation and growth of OSCC,

consequently varying its expression according to the region analyzed. This fact, in addition to different proportions between lesional parenchyma and stroma, as well as the amount of inflammatory infiltrate, and glandular and vascular cells in the stroma of the samples, justifies the high standard deviation observed in the Western blot analysis. Interestingly, the present study showed that samples from ex-drinkers patients expressed significantly more Runx1 and ETV5, while samples from patients that currently consume alcohol and tobacco expressed significantly more Runx1 only. While smoking and alcohol consumption are important etiologic agents of OSCC, these results suggest that Runx1 and ETV5 may be involved in the malignant transformation process. To the best of our knowledge, this is the first study that correlates transcriptions factors with alcohol and tobacco consumption.

Initially, in the present study, it was hypothesized that Runx1 expression would be confined to the cytoplasm, while ETV5 would be mainly nuclear. However, if carefully analyzed in high magnifications, it is possible to observe the nuclear staining of Runx1. In addition, the immunofluorescence confirmed the granular staining for both factors in the nucleus of parenchymal cells. The immunofluorescence of Runx1 was very similar to the one observed in OSCC cell lines in the study of Scheitz et al. (2012) [10]. Runx1 regulates several genes that participate in SCC development, i.e., activating Stat3 and repressing p21, making Runx1 one of the central players in SCC formation [25]. Furthermore, as in hematopoietic processes, Runx1 interacts with other transcription factors in solid tumors, as ETV5 in endometrioid and ovarian carcinoma, culminating in disruption of epithelial BM through the high expression of MMPs 2 and 9 in the invasive front [19]. ETV5 has a significant role in regulating of MMP-2 expression in human chondrosarcoma, contributing to tumor progression and invasion [23]. Furthermore, Runx1 and ETV5 are correlated with early development of endometrioid carcinoma [20]. Taking into account, it is suggested that these transcription factors play important roles in the OSCC development.

Although zymography has been extensively used for indicating gelatinolytic activity and expression of these MMPs, the interpretation of the zymography was performed with more carefully in the present study, considering the results only as expression of MMPs. In zymography, MMPs are artificially activated; therefore, the activity observed in the gel may not represent the real activity that occurs in the original tissue location. SCC samples expressed significantly more MMP-9 than others samples, while MMP-2 expression was relatively constant in all samples. Not considering the clinical or microscopic diagnoses, the samples from tobacco-consuming patients expressed MMP-9 significantly more, in agreement with Renò et al. (2011) that showed that chronic exposure to tobacco induces higher basal expression of MMP-2, -9 and -28 in oral keratinocytes [52]. In immunohistochemistry, the

strong expression of MMP-2 in the parenchyma of Control and HA are in agreement with Mäkelä et al. (1999), that suggested that MMP-2 is involved in the physiological migration of keratinocytes of the oral mucosa [53]. MMP-9 was weakly expressed in the basal layer of epithelium and stroma of SD samples, while expression was increased in some tumoral cells and mainly in the stroma that surrounded the tumoral nests of SCC samples, reinforcing the study of Sobral et al. (2011) that suggested that stromal myofibroblasts in OSCC promote invasion throughout secretion of stromal MMP-9 [33].

It was observed a strong positive correlation between Runx1 and ETV5 expressions, which approximately 58% of SD and 53% of SCC samples co-expressed Runx1 and ETV5 mainly in the nuclear neoplastic cells. These samples expressed significantly more MMP-2 and -9, and presented points of discontinuous epithelial BM. These finds reinforce the hypothesis that Runx1 and ETV5 together stimulate the expression of MMP-2 and -9, contributing to the disruption of epithelial basal membrane. It is know that loss of *Runx1* shrinks tumours in mice and impairs human and mouse tumour cell growth *in vitro*, but is not essential for normal oral epithelium homeostasis. In addition, the Runx1-positive cells are long-lived with great self-renewing, which can give them a greater potential to be stem cells. Taking into account, Runx1 could be a promising target for treatment and prevention of epithelial neoplasias [10]. The results of present study reinforce this hypothesis and suggest that Runx1 and ETV5 can be target for treatment and prevention of OSCC.

This is the first study that characterized the expression of Runx1 and ETV5 transcription factors in normal oral mucosa, oral leukoplakia and oral squamous cell carcinoma of humans. The fact that both transcription factors are more expressed and correlated with higher expression of MMP-2 and -9, disruption of epithelial BM and higher cellular proliferation index in severe oral epithelial dysplasia and oral squamous cell carcinoma, suggest that Runx1 and ETV5 play an important role in the oral squamous cell carcinoma development.

MATERIALS AND METHODS

Ethical Approach

This research was approved by the Institutional Review Board (IRB) of the Piracicaba Dentistry School (UNICAMP).

Patients and Samples Preparation

This was a prospective study performed with frozen samples obtained from biopsies of lesions with clinical suspicion of OSCC and/or OL. OLs were clinically classified in homogeneous and non-homogeneous. The normal oral mucosa (control) was obtained from borders of oral fibrous hyperplasia. Smoking and alcohol consumption habits were assessed for every patient.

A small fragment was collected from fresh biopsy specimens immediately after the biopsy procedure. It was sectioned in the middle, immersed on O.C.T. (Tissue-Tek®), quickly frozen on dry ice and stored in an -80ºC freezer [54]. Posteriorly, the frozen samples were sectioned in a cryostat (CM1850, Leica Microsystems, Germany) in two manners: 1) Five or more sections with 30µm of each sample were obtained and stored in a 2 ml tube at -80ºC in a freezer for subsequent protein extraction; 2) Ten glass slides (silane-coated) with two 5-µm-thick sections were made and stored at -20ºC. These slides were used in the hematoxylin and eosin stains (HE) and in the immunohistochemistry and immunofluorescence reactions.

Microscopic Diagnosis and Selection Criteria

An experienced oral pathologist performed the diagnosis of each sample examining an HE slide from each sample. The diagnosis obtained was compared with the diagnosis obtained in biopsy (paraffined material). Only the samples that: 1) Presented correspondent diagnosis with the paraffined material; 2) Obtained the diagnosis of epithelial dysplasia, squamous cell carcinoma or normal mucosa (control) were included in the study.

Grading Criteria of Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma

Considering the inherent variation on microscopic grading in borderline cases, it was decided to classify the samples according to an adaptation of WHO criteria [7]. The oral epithelial dysplasias were classified in 03 groups: Hyperkeratosis and Acanthosis (HA), Mild/Moderate Epithelial Dysplasia (MMD) and Severe Epithelial Dysplasia (SD). While oral

squamous cell carcinomas (OSCC) were classified in 02 groups according with their differentiation: Mild/Moderate Differentiated OSCC and Poorly Differentiated OSCC.

Protein Extraction

The tubes with the 30-µm-thick frozen sections were removed from the -80ºC freezer and immediately immersed in ice for slow defrosting. The samples were washed three times for 10 minutes to remove blood and OCT. The baths were followed by centrifugation at 10,000 rpm for 10 minutes at 4°C in refrigerated centrifuge (5417R, Eppendorf, Germany) for tissue precipitation. Protein extraction was performed by RIPA Buffer (R0278, SIGMA-ALDRICH, USA) with protease inhibitor (cOmplete™ Mini, 11836153001, Roche, USA) and phosphatase inhibitor (PhoStop, 4906845001, Roche, USA) diluted according to the manufacturer's instructions. The extraction was optimized by utilization of 0.1 mm Zirconia/Silica Beads (11079101z, Biospec Products, USA) and Mini Beadbeater 8 (Biospec Products, USA) two times per sample for 1 minute each. The cycles were followed by cooling the samples in ice for 1 minute, centrifuged in 14000 rpm at 4ºC for 10 minutes and the resulting protein extract was collected and kept in ice. The protein concentration was determined by Pierce™ BCA Protein Assay Kit (23225, Thermo Ficher Scientific, USA) and Spectrophotometer GENESYS™ 10 UV (Thermo Scientific, USA) used according to the manufacturer's instructions. The protein extracts were stored in an -80ºC freezer.

Western blot for Runx1 and ETV5

Thirty µg of total protein extract per sample were resolved in a 12% Precast Protein Gel (Mini-PROTEAN® TGX™, 4561043, Bio-Rad, USA) electrophoresis (SDS-PAGE) under reducing conditions, and transferred onto nitrocellulose membranes (Amersham Protran Premium 0.45 NC, 29047575, GE Healthcare Life Sciences). The membranes were blocked for 12 hours in 10% milk in PBST (PBS containing 0.1% Tween 20) and washed in PBST three times for 10 minutes each. The primary antibodies used were identical to the immunohistochemistry in a 1:1000 concentration in 5% non-fat dry milk in PBST. The incubation lasted two hours. After three baths in PBST, the membrane was incubated with secondary antibody Anti-Mouse IgG Alexa Fluor® 790 Conjugated (Donkey Polyclonal Antibody, ab186699, Abcam, UK), washed 3 times in PBST and developed using the UVITec Alliance 4.7 (Cambridge, UK). Ten µg of Jurkat Cell Lysate (12-303, EMD Millipore, USA) was used as positive control in each reaction. The loading control per sample was the Anti-Beta Actin Alexa Fluor® 790 Conjugated (Mouse Monoclonal Antibody, clone mAbcam 8226, ab184576, Abcam, UK). The intensities of positive bands were determined using the

Image Studio™ Lite Software (Version 5.2, LI-COR Biosciences, UK) and all results were normalized by intensities of their respective loading controls [55].

Zymography

Ten µg of total protein extract per sample were mixed with non-reducing sample buffer and resolved in 10% sodium dodecylsulfate-polyacrilamide gels (SDS-PAGE) copolymerized with 1.6 mg/mL of gelatin (G8150, SIGMA-ALDRICH, Germany) as substrate. Following renaturation of the proteins by incubating the gels two times in a 2% Triton X-100 (T9284, SIGMA-ALDRICH, Germany) solution for 20 minutes each at room temperature, the gels were immersed in the activation buffer (50 mM Tris–HCl pH 7.4, 5 mM CaCl2) for 16 hours at 37ºC. Gelatinolytic activities were detected after staining with Coomassie Brilliant Blue R250 (1610400, Bio-Rad, USA). MMP activities were confirmed by adding 2 mM of 1.10-phenanthroline (E12055, InvitrogenTM, USA) to the activation buffer. The intensity of the negative bands were obtained and determined by the UVITec Alliance 4.7 and the Image Studio™ Lite Software, respectively.

Immunohistochemistry for Runx1, ETV5, MMP-2 and MMP-9

These reactions were performed in the frozen samples. The glass slides were removed from the -20ºC freezer and immediately immersed in PBS (Phosphate-buffered saline) two times for 5 minutes each. The endogenous peroxidase activity was blocked with 10% H2O2 for 10 minutes and then washed five times for 30 seconds each, followed by 2 hours of incubation with the primary antibodies Anti-Runx-1 (1:200, Mouse Monoclonal Antibody, clone 5A1, MABD169, Millipore EMD, USA), Anti-ETV5 (1:200, Mouse Monoclonal Antibody, clone 3H3, MABN683, Millipore EMD, USA), Anti-MMP-2 (1:200 Antibody, Rabbit Polyclonal, AB19167, Millipore EMD, USA) and Anti-MMP-9 (1:200, Rabbit Monoclonal Antibody, clone EP1254, 04-1150, Millipore EMD, USA). All primary antibodies were diluted in an Antibody Diluent with Background Reducing Components (S3022, Dako, Glostrup, Denmark). The secondary antibody was the conjugated with polymer dextran marked with peroxidase (Dako EnVision Labeled Polymer; Dako, Glostrup, Denmark) used according to the manufacturer's instructions. Reactions were developed with a solution containing 0.6 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) and 0.01% H2O2 and counterstained with Carazzi’s hematoxylin. Sections of frozen human placenta were included in all reactions as positive control for all antibodies. Negative controls of reactions were performed by omitting the primary antibody in the second section of the same glass slide. Cytoplasmic and nuclear stains were considered positive. The

analysis of results was only descriptive and representative samples were photographed by an optical microscope (DMR, Leica Microsystems, Germany) attached to a digital camera (DFC 450, Leica Microsystems, Germany).

Immunofluorescence

The double immunofluorescence assay was performed for Runx1 (1:50, Rabbit Polyclonal Antibody, S276, ABGENT, USA) and ETV5 (same of immunohistochemistry). Briefly, the glass slides were removed from -20ºC freezer and immediately immersed in PBS two times for 5 minutes each. The primary antibodies were incubated for 2 hours. The slides were washed in PBS two times for 5 minutes each and then incubated with the secondary antibodies Anti-Mouse IgG FITC Conjugate (1:200, Goat Polyclonal Antibody, AP181F, EMD Millipore, USA), Anti-Rabbit IgG Rhodamine Conjugate (1:200, Goat Polyclonal Antibody, AP187R, EMD Millipore, USA). The sections were washed in PBS two times for 5 minutes each and mounted with VECTASHIELD® (Mounting Medium with DAPI, H-1200, Vector Laboratories, USA). For immunofluorescence for type IV collagen, the primary antibody Anti-collagen IV (1:100, Mouse Monoclonal Antibody, MAB1430, EMD Millipore, USA) and the secondary antibody Anti-Mouse IgG AMCA Conjugate (1:200, Goat Policlonal Antibody, AP181M, EMD Millipore, USA) were used in the same protocol described above. In this case, the slides were mounted with VECTASHIELD® HardSet (Antifade Mounting Medium without DAPI, H-1400, Vector Laboratories, USA). All primary and secondary antibodies were diluted in an Antibody Diluent with Background Reducing Components (S3022, Dako, Glostrup, Denmark). Sections of frozen human placenta were included in all reactions as positive control for all antibodies. Negative controls were performed by omitting the primary antibody in a second section of the same glass slide. The representative samples were photographed by a fluorescence microscope (DMR, Leica Microsystems, Germany) attached to a digital camera (DFC 345FX, Leica Microsystems, Germany). The images obtained for double immunofluorescence were overlapped by Picasa 3 software (Google). The analysis of results was descriptive.

Cellular Proliferation Index

The cellular proliferation index was determined by Ki-67 immunohistochemistry quantification. The reactions were performed in the correspondent formalin-fixed and paraffin-embedded tissues of the frozen samples. The 3-µm-thick sections mounted on silane-coated glass slides were de-paraffinized in xylene and rehydrated in graded ethanol solutions. The antigen retrieval was performed with EDTA/Tris buffer (pH 9.0) in a pressure cook,

followed by inhibition of endogenous peroxidase activity by 10% H2O2 (five cycles of 5 minutes each). After washing in PBS buffer (pH 7.4), slides were incubated overnight with primary antibody anti-Ki67 (1:100, Mouse Monoclonal Antibody, clone MIB1, Dako Cytomation, USA) diluted in an Antibody Diluent with Background Reducing Components (S3022, Dako, Glostrup, Denmark). All slides were subsequently exposed to avidin-biotin complex and horseradish peroxidase reagents (LSAB Kit, K067511, DakoCytomation, USA) and diaminobenzidine tetrahydrochloride (DAB, Sigma, USA). Finally, the slides were counterstained with Carazzi hematoxylin. Positive control sections were used for each reaction, whereas the negative control was obtained by omitting the specific primary antibody. The immunohistochemical slides were subsequently scanned into high-resolution images using the Aperio Scanscope CS® Slide Scanner (Aperio Technologies Inc., Vista, CA, USA). All digital images obtained were analyzed using ImageScope software (Aperio Technologies Inc., Vista, CA, USA). Ki67 nuclear staining was analyzed using the Nuclear V9 Algorithm (Aperio Technologies Inc., Vista, CA, USA) with the following input parameters: averaging radius 1.0, curvature threshold 3.0, lower threshold 0, upper threshold 230, minimum nuclear size 30.0, maximum nuclear size 124.0, minimum roundness 0.2, minimum compactness 0.25, minimum elongation 0.1, clear area objective 250, and an intensity threshold ranging from 0 to 200, where strong staining was considered from 0 to 160 and weak staining from 160 to 200. Approximately 1000 epithelial cells in total of hotspot areas were quantified in each sample. The percentage of positive cells was used as cellular proliferation index [56]. The representative samples were photographed.

Statistical Analysis

Statistical significance was calculated using Mann-Whitney U test or one-way analysis of variance (ANOVA) based on Bonferroni's multiple comparisons. Correlations analyses were performed using Spearman's rank correlation. A P value of less than 0.05 was considered statistically significant and all the P values were two-tailed. Data are shown as mean and standard deviations.

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CONFLICTS OF INTEREST

None

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3 CONCLUSÃO

Este é o primeiro estudo que caracterizou e correlacionou a expressão dos fatores de transcrição Runx1 e ETV5 em mucosa normal, leucoplasia e carcinoma espinocelular da cavidade oral de seres humanos. O fato de ambos serem mais expressos e correlacionados com maior expressão das MMPs 2 e 9, ruptura da lamina basal epitelial e maior índice de proliferação celular em displasias epiteliais intensas e carcinomas espinocelulares orais, sugere que o Runx1 e ETV5 desempenham um importante papel no desenvolvimento do carcinoma espinocelular da cavidade oral.

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[1] De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

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Anexo 01: Certificado do Comitê de Ética em Pesquisa FOP/Unicamp

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Correlação da expressão dos fatores de transcrição RUNX1 e ETV5 com a expressão e atividade das metaloproteinases 2 e 9 em lesões potencialmente malignas e carcinomas espinocelulares bucais"**, protocolo nº 157/2014, dos pesquisadores Marcondes Sena Filho, Jacks Jorge Junior, Oslei Paes de Almeida e Pablo Agustin Vargas, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 12/08/2015.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"Correlation of the RUNX1 and ETV5 expression with the expression and activity of the metalloproteinases 2 and 9 in oral potentially malignant lesions and oral squamous cell carcinomas"**, register number 157/2014, of Marcondes Sena Filho, Jacks Jorge Junior, Oslei Paes de Almeida and Pablo Agustin Vargas, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on Aug 12, 2015.

Profa. Dra. Fernanda Miori Pascon
Secretária
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

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Manuscript #	011377
Current Revision #	0
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Title	The transcription factors Runx1 and ETV5 are correlated with high expression of metalloproteinases 2 and 9 in severe oral epithelial dysplasia and oral squamous cell carcinoma
Manuscript Type	Research Paper
Corresponding Author	Marcondes Sena-Filho (Piracicaba Dental School - University of Campinas)
Contributing Authors	Rogerio Jorge , Marcio Lopes , Pablo Vargas , Raquel Gerlach , Oslei Almeida , Jacks Jorge
Abstract	Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck region. The mucosal changes that precede the occurrence of OSCC are of great interest in this context, in which the oral leukoplakia (OL) is a subject of several studies. Runx1 is a transcription factor involved in physiological and pathologic events of hematopoiesis, but its functions in solid tumors is still poorly understood. Some studies have suggested a possible interaction of Runx1 with others transcription factors, as ETV5, stimulating the production of matrix metalloproteinases (MMP) 2 and 9 in some solid malignancies, as endometrioid carcinoma and chondrosarcoma. Through immunohistochemistry, immunofluorescence, Western blot and zymography, this study evaluated and correlated the expression of Runx1 and ETV5 with the expression of MMP-2 and MMP-9, epithelial basal membrane integrity and cellular proliferation index in OL, OSCC and normal oral mucosa. The results demonstrated that the expression of Runx1 and ETV5 are correlated with high expression of MMPs 2 and 9, disruption of epithelial basement membrane and high proliferation index in OL with severe epithelial dysplasia and OSCC, which suggest that Runx1 and ETV5 play an important role in the oral squamous cell carcinoma development.
Keywords	Oral Carcinoma, Oral Leukoplakia, Potentially Malignant Disorders, AML1, ETS
Conflict of Interest	**No**, there is no conflict of interest that I should disclose, having read the above statement.